UNIVERSITI PUTRA MALAYSIA

SEROLOGICAL SURVEY OF EQUINE INFECTIOUS ANEMIA VIRUS (EIAV) IN HORSES IN SELANGQR

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BY

ABDUL NASSER ASHOUR ALTAEB

A project paper submitted to Faculty of Veterinary Medicine, Universiti Putra Malaysia in Partial Fulfillment of the Requirements for the Degree of Master of Veterinary Medicine (M.V.M)

UNIVERSITI PUTRA MALAYSIA
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MALAYSIA

March 2004

DEDICATION

To MY PARENTS, MY WIFE, MY SON HAMAM AND MY BROTHERS

AND SISTERS

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, Universiti Putra Malaysia in partial fulfillments of the requirements for the degree of Master of Veterinary Medicine

SEROLOGICAL SURVEY OF EQUINE INFECTIOUS ANEMIA VIRUS (EIAV) IN HORSES IN SELANGOR

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March 2004

Supervisor: Dr. Mohd Azam Khan Goriman Khan

Faculty: Veterinary Medicine

A study was carried out to determine the prevalence of Equine Infectious Anemia Virus (EIAV) among horses of various clubs in the state of Selangor. A total of 94 serum samples was obtained from horses of various breeds, sex and age and tested for the presence of antibodies against EIAV using; (i) competitive enzyme-linked immunosorbent assay (CELISA) and (ii) agar gel immunodiffusion (AGID) test, to demonstrate the presence of antibodies against p26 recombinant gag protein. All horses sampled in this study were kept in stables and have access to paddock grazing. Horses have no history of contact with imported stocks. In this study none of the sera had antibodies against EIA virus. It is possible that the strict import policies imposed, and the almost non-existent importation of horses for the purpose of breeding has prevented the introduction of the disease into Malaysia.

ABSTRAK

Abstrak daripada kertas projek yang dibentangkan
kepada Fakulti Perubatan Veterinar, Universiti Putra Malaysia adalah sebahagian
daripada keperluan memenuhi Ijazah Sarjana dalam Perubatan Veterinar

KAJI SELIDIK SEROLOGI TERHADAP VIRUS ANEMIA BERJANGKIT KUDA (EIAV) KE ATAS KUDA-KUDA DI SELANGOR

Oleh

ABDUL NASSER ASHOUR ALTAEB

Mac 2004

Penyelia : Dr. Mohd Azam Khan Goriman Khan

Fakulti: Perubatan Veterinar

Satu kajian telah dijalankan untuk mengkaji prevalens virus anaemia berjangkit ekuin (EIAV) di kalangan kuda-kuda dari berbagai kelab di negeri Selangor. Sejumlah 94 sampel serum telah diambil dari kuda-kuda pelbagai baka, jantina dan umur, dan telah diuji untuk menentukan kehadiran antibodi-antibodi terhadap EIAV dengan menggunakan (i) asai imunoerap terangkai enzim bersaing (CELISA) dan (ii) ujian imunoresapan gel agar-agar (AGID) untuk menunjukkan kehadiran antibodi-antibodi terhadap rekombinan protein gag p26. Semua kuda-kuda yang di sampel dalam kajian ini disimpan di dalam kandang dan dibenarkan melakukan ragutan alongan. Kuda-kuda terlibat tidak pernah di dalam sejarah bersentuh dengan stok-stok yang di import. Di dalam kajian ini tiada satu pun serum-serum yang diuji mengandungi antibodi terhadap virus EIA. Kemungkinan polisi-polisi ketat pengimportan yang dikuatkuasakan dan hampir tiadanya pengimportan kuda-kuda untuk tujuan pembiakan, telah mencegah kemasukan penyakit tersebut ke Malaysia.

APPROVAL

It is hereby certified that we have read this project paper entitled "Serological Survey of Equine Infectious Anemia Virus (EIAV) in Horses in Selangor" by Abdul Nasser Ashour Altaeb and in our opinion; it is satisfactory in terms of scope, quality and presentations as fulfillment of the requirement for the degree of Master of Veterinary Medicine, VPD 5908 project.

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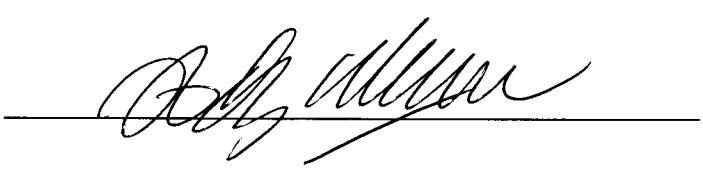

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My sincere thanks are also extended to the entire faculty; post graduate students, my friends, and technicians at Biologics Laboratory.

Finally, my appreciation and my utmost gratitude are expressed to my wife, for her support and encouragement and to my parents for their prayers.

DECLARATION

I hereby declare that the project paper is based on my original work except quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at UPM or other institutions.

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Abdul Nasser Ashour Altaeb
Date…

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
BIV	Bovine immunodeficiency virus
CA	Mature capsid
CAEV	Caprine arthritis encephalitis virus or Visna virus
CDC	Center for disease control
CF12h	Canine osteosarcoma cells
CF	Complement fixation test
ED	Equine dermal cell line
EIAV	Equine infectious anemia virus
FEA	Feline embryonic cells
FEK	Fetal equine kidney cell
FIV	Feline immunodeficiency virus
FP	Fluorescence polarization test
gp	Glycoprotein
HIV	Human immunodeficiency virus
HI	Hemagglutination inhibition
ID	Immunnodiffussion test
IN	Integrase
LTR	Long terminal repeats
MA	Matrix
NC	Nucleocapsid
OIE	Office International des Epizooties

PCR	Polymerase chain reaction
R T	Reverse transcriptase
SIVS	Simian immunodeficiency viruses
SN	Serum neutralization test
SU	Env gene encodes the surface
TM	Transmembrane
U3	Unique 3
U5	Unique 5
WHO	World Health Organization
WY	Wyoming strain

CHAPTER 1

INTRODUCTION

Equine infectious anemia (EIA) or Swamp Fever is a multisystemic retroviral disease of equidae, characterized by hemolytic anemia that is immune mediated (Clabough, 1990). The disease is caused by Equine infectious anemia virus (EIAV), a member of the lentivirus subfamily of retroviruses. The virus possesses the surface glycoproteins, gp90 and gp45 and four major nonglycosylated internal core proteins, known as p26, p15, p11 and p9 (Parekh *et al.*, 1980; Montelaro *et al.*, 1984; Hussain *et al.*, 1988) (Appendix 2).

The disease is distributed worldwide in the Equidae family; and has been reported in horses, donkeys and mules (Montelaro *et al.*, 1984; Hammond *et al.*, 1997). Once an animal is infected, it will remain infected for the rest of its life (Montelaro *et al.*, 1984; Clements and Zink, 1996; Issel *et al.*, 1982).

After infection, the animal experiences three distinct disease phases: acute, chronic and inapparent carrier (Clabough, 1990; Roberts and Lucas, 1987). The acute stage of infection is characterized by recurrent febrile episodes with high virus load. After resolution of the primary viremia, most animals develop chronic EIA, which is characterized by recurrent cycles of the disease, associated with weight loss and anemia, leading to a prolonged and symptomatic period, and finally an inapparent carrier stage for the rest of the animal's life (Hammond *et al.*, 1997).

Most infected horses develop a vigorous humoral immune response, the envelope glycoproteins being the major immunogens. Each episode of viremia is associated with the appearance of a new and predominant antigenic variant of the virus; caused by point mutations in the env gene that codes the viral surface proteins (Montelaro *et al.*, 1984).

Although EIA infection persists life-long, during non-fibrile intervals the likelihood of isolating EIA from blood samples is close to nil. The diagnosis of EIA rests entirely on serological evidence (Issel *et al.,* 1988). Coggins *et al.*, (1972) developed an agar gel immunodiffusion (AGID) test to detect precipitating antibodies to the core protein p26, which is known to be group-reactive and antigenically stable (Payne *et al.*, 1984). The AGID test is the only test officially recognized by the Office International des Epizooties (OIE) in Paris (Pearson and Coggins, 1979). A positive AGID test noted 15 to 25 days post infection (Coggins *et al.*, 1972), confirms that the virus is present as infected horses produce antibodies to EIAV proteins within 12 days of infection (Issel and Cook, 1993).

Enzyme-linked immunosorbent assay (ELISA) was developed in the late 1980's for faster and more sensitive serodiagnosis (Issel *et al.,* 1988; Archambault *et al.*, 1989; Ramachandran *et al.,* 1990). Competitive ELISA (CELISA) was developed using purified antigens from cell culture adapted EIAV strain Wyoming, to detect antibodies against p26 protein. Although the authors found a good correlation between AGID and CELISA, the latter could not detect weak positive antibody titers (Burki *et al.*, 1992; Issel and Cook, 1993). Another ELISA was developed using the amino-terminal portion of a recombinant gp45 protein as antigen

(Hancock and Tsang, 1986; Archambault *et al.*, 1989; Thomas *et al.*, 1991; Lew *et al.*, 1993). Soutullo *et al.*, (2001) designed an ELISA test using linear and cyclic synthetic peptides from gp45 and gp90 as antigen. To date, the largest amount of serological information relating to the presence of EIAV infection has been obtained using the internal core protein p26 and surface glycoprotein gp90 and gp45 isolates as the antigens.

Since clinical diagnosis is difficult for acute and inapparent infection, the movement of horses across borders stands the risk of causing economic embarrassments and interference to sporting events due to EIA. Economic losses due to the disease can be large as there is no treatment or vaccination, for this disease. Mortality rates may approach 50% and the athletic ability of horses reduced.

Serologic evidence for EIAV infection has been reported in many countries around the world. Using AGID and CELISA the prevalence of this disease was 1.5-2.5% in the USA, 6% in Canada, a low level in France, 1.6% in West Germany, and 15-25% in Argentina and 50% in Brazil. The disease has never been recorded in Malaysia. Large-scale movements of horses during wartime have been responsible for extensive dissemination of the disease. At the present time there is another surge of infection in most countries, possibly due to obligatory testing conducted as surveillance of the disease. It is suggested that the rapid expansion of 'pleasure horses' activity in affluent countries leads to more movement and opportunities for spread of the infection from relatively few donors (Coggins, 1984).

Since there are no records on EIAV seroprevalence among horses in Malaysia, the objective of this study was to investigate the seroprevalence of Equine Infectious Anemia (EIA) among horses in Selangor using two different methods, Competitive Enzyme Linked Imunosorbent Assay and Agar Gel Immunodiffusion (Coggins Test).

CHAPTER 2

LITERATURE REVIEW

2.1 Retroviruses

In 1981 the center for disease control (CDC) USA, first described the symptoms of disease that would be identified later as acquired immune deficiency syndrome (AIDS). AIDS caused by a retrovirus infect other animals such as rodents, monkeys, and horses. Nineteen years after the isolation of the human immunodeficiency virus (HIV), the etiology agent of AIDS, the World Health Organization (WHO) estimates 65 million people to be infected with HIV world wide, thus establishing HIV as the leading cause of death by infectious agents.

The impact of HIV has greatly changed the direction of scientific research and brought the field of retrovirology to the forefront of medicine. The Retroviridae family includes viruses with a diploid RNA genome that utilizes reverse transcriptase, an RNA dependent DNA polymerase. Retroviruses are enveloped viruses ranging in size from 80–100 nm in diameter and contain the enzymes reverse transcriptase integrase and protease (Coffin *et al.,* 1997).

2.2 Retrovirus Genome and Proteins

As reviewed by Coffin *et al.*, (1997) the retrovirus family can be divided in two main groups, based on genome organizations, simple and complex; Simple retroviruses have gag, pro, pol and env genes that encode polyproteins. The gag gene encodes the mature capsid (CA), matrix (MA), and nucleocapsid (NC)

structural proteins. The pol encodes reverse transcriptase (RT), and integrase (IN). The gag and pol polyproteins are proteolyticlally cleaved via viral protease, encoded by the Pro gene. The env gene encodes the surface (SU) and transmembrane (TM) outer structure proteins. The env polyprotein is cleaved by host cell proteases. The retrovirus provirus also contains non-coding long terminal repeats (LTR) located at the 5'and 3' ends. The LTR consists of U3 (unique 3') and U5 regions. The U3 element occurs once at 3' end of viral genomic RNA and twice in proviral DNA. The U3 region is between 120 – 1200 nucleotides in length.

2.3 Lentiviruses

The term lentivirus originates from the Latin world lenti meaning slow. This sub group of retroviruses is named due to the long incubation period from initial infection with the virus, to appearance of disease symptoms. Viruses in the lentiviruses group of retroviruses are the human immunodeficiency virus (HIV–1 and HIV–2), simian immunodeficiency viruses (SIVS), equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV), visna virus or caprine arthritis encephalitis virus (CAEV), and feline immunodeficiency virus (FIV). EIAV and other lentiviruses encods a dUTPase enzyme (designated DU), which maps to the pol gene (McClure *et al.*, 1988: Elder *et al.*, 1992).

2.4 Equine Infectious Anemia Virus (EIAV)

In vitro, several strains of EIAV have been shown to infect and replicate in feline embryonic cells (FEA), canine osteosarcoma cells (CF12h), and equine macrophages. Strains of EIAV exhibit differing cellular tropism. The Wyoming strain (WY) is a highly virulent wild type strain of EIAV, and its replication *in vitro*

is restricted to equine macrophages in which it replicates to high titers. The WY strain was adapted to replicate in ED, FEK, FEA and CF12h cell lines and was termed the Malmquist strain (Malmquist *et al.*, 1973). EIAV differs from other human and non-human lantiviruses *in vivo* in that it has not been found to infect lymphocytes; it is predominantly macrophage tropic (Clabough, 1990; Issel *et al.*, 1988). Pathogenically, EIA differs in several respects from other lentivirus infections, in that it does not induce direct immunosuppression (Montelaro *et al.*, 1989), but circulating immune-complexes (Henson *et al.*, 1973).

2.5 Epidemiology of the Disease

2.5.1 Occurrence

EIA has been diagnosed in several different continents. In Europe, it is most prevalent in the northern and central regions. It has appeared in most states in the United States and the provinces of Canada but the principal enzootic areas are the Gulf Coast region and the northern wooded sections of Canada (Paquette, 1985). Diagnosis of the disease was made in Australia in 1959, but the incidence appears to be very low (Lepherd, 1981). The only area of Australia in which EIA could be regarded as being endemic is along the inland river systems of central and western Queensland. In a serological survey in this area in 1978, 21.7% of horses and 23% of properties were positive for EIAV (Thomas and Elder, 1978). The disease was also reported once in Thailand in 1996 and Mongolia (Vallat, 2003) (Appendix 1).

The morbidity varies considerably and depends on the strain of the virus, and the inoculum delivered by the biting insects (Coggins, 1984). Extensive serological surveys over large areas, using the agar gel immunodiffusion (AGID) Coggins test have shown the prevalence rates, ranging from 1.5 – 2.5 % in the United States, to

50% in Brazil (Jenner and Romulo, 1994). The prevalence of infection varies depending on the population of horses, the proportion of carrier and the density of insect vectors (Hall *et al.*, 1988). Large-scale movements of horses during wartime have been responsible for extensive dissemination of the disease. The possible detection of the infection at present is due to obligatory testing carried out. Rapid expansion of 'pleasure horse' activity in affluent countries may lead to more movement of horses and opportunities for spread of the infection from relatively few donors (Coggins, 1984).

2.5.2 Methods of Transmission

EIA virus is relatively resistant to most disinfectants and cannot be destroyed by boiling for 15 minutes, but is destroyed by sunlight. It persists for several months at room temperature in urine, feces, dried blood and serum. It is present in all tissues, secretions and excretions and may persist in the body for up to 18 years, providing a source of infection for most of the animal's life (Issel *et al.*, 1988). Horses that are febrile and show clinical signs of EIA have a higher titer of viremia and much more likely to serve as a source of disease transmission than inapparent carriers (Kono, 1969; Issel *et al.*, 1982; Orrego, 1983).

Infection by the EIA virus usually occurs by transmission of blood between infected and uninfected horses, although body secretions may contain virus and could serve as a source of virus (Tshjian *et al.*, 1984). Short – term contact is usually insufficient to cause spread, but continued, close association with susceptible animals usually results in infection. Spread within a group is slow, although occasionally fairly rapid spread is observed in large groups of horses assembled at racetracks or army depots (Clabough, 1990).

Insect vectors, the primary mode of transmission, with disease passage occurring through interrupted feeding of stable fly (*Stomoxys calcitrans*), deer flies (*Chrysops spp.*) and horse flies (*Tabanus spp.* and *Hypomirta spp.)* (Stein *et al.,* 1942; Hawkins *et al.*, 1973, 1976; Kemen *et al.*, 1978; Cupp and Kemen, 1980; Issel *et al.*, 1982; Foil *et al.*, 1983; Issel and Foil, 1984). The disease spreads most actively in summer and in marshy or wooded areas, suggesting that bites by insects may be the most important method of spread. It is probable that transmission is mechanical only (Cupp and Kemen, 1980; Issel and Foil, 1984). Therefore, wild type EIAV will not multiply in any species of insects (Shen *et al.*, 1978; Williams *et al.*, 1981).

The virus has been transmitted under experimental and natural conditions from inapparently infected animals (Issel *et al.*, 1982; Foil *et al.*, 1988) where viremia titters are substantially lower than those observed in horses with clinical EIA (Kono *et al* 1976; Issel *et al.*, 1982). In enzootic areas, outbreaks have been caused by the use of untreated biological preparations of equine origin (Issel *et al.*, 1988).

Kemen and Coggins (1972) have reported intrauterine transmission. Foals have reportedly become infected through the milk of infected dams, but relatively large amounts of virus must be ingested to cause infection and the digestive tract is not a major portal of entry. Foals of infected dams are less susceptible to natural infection than adults possibly due partly to persistence of colostral antibodies (Foil *et al.*, 1983). The opposite can also happen and the passively immune foal develops a fatal case of the acute disease (Issel *et al.*, 1985).

2.5.3 Risk Factors

2.5.3.1 Environmental Factors

There are marked seasonal incidence of the disease, most cases occurring in the summer and autumn. This is associated with low-lying and newly settled bush area and due to greater number of the insect vectors in such areas (Issel *et al.*, 1992). Distances between horses are important to the carrier. Although tabanids are strong flyers, they usually prefer to complete an interrupted meal on the initial host or a nearby host (Foil, 1983). Prolonged therapy with corticosteroids and poor environment or management is known to induce recrudescence of EIA (Kono *et al.*, 1976).

2.5.3.2 Animal Factors

All breeds and age groups of equidae are susceptible to EIAV. The indigenous Criollo horses of Argentina are reported to be much more resistant to infection and only mildly affected by the disease (Shen *et al.*, 1984). Foals have lower tabanid burdens than older horses due in part to their more vigorous defense movements (Issel *et al.,* 1985; Foil *et al.*, 1985).

2.6 Pathogenesis

After infection EIAV multiplies in tissues that have abundant macrophages, notably the liver, spleen, lymph nodes, lungs and kidney (Issel *et al.,* 1988; Clabough 1990) as viral replication occurs only in mature tissue of macrophages and does not occur in circulating monocytes (Maury 1994; Sellon *et al.,* 1992). The acute form of the disease is thought to be associated with massive virus replication in and destruction of macrophages, but the actual cause of death is unknown. There is good

evidence that the vascular lesions and the erythrocyte fragility are part of an immune reaction (Clabough 1990; Issel *et al.*, 1988).

2.7 Immune Reaction

The immune response to EIAV is responsible for controlling replication of the virus and also plays an important role in the pathogenesis of the disease. The major clinical signs and lesions of EIA are attributable to the host response to the virus and not direct viral damage to the tissue (Sellon, 1993). Replication of EIAV stimulates a strong immune response that is detectable within 7 to 10 days of infection. Antibodies to the p26 core protein are detectable by AGID test in almost all horses 45 days after infection and by 60 days after infection antibodies to gp45 and gp90 are present (Clabough, 1990). The immune response includes the production of virus neutralizing antibodies, complement-fixating antibodies, and cytotoxic T lymphocytes (McGuire *et al.*, 1994). The immune responses are responsible for the termination of viremia, but this effect is not mediated by antibody-dependent cellular cytotoxicity against EIAV- infected macrophages (Tschetter *et al.*, 1997). Virus-antibody complex are readily phagocytosed by cells of the reticulo-endothelial system, including tissue macrophages, and are involved in the development of the fever, depression, thrombocytopenia, anemia and glumerulonephritis, which are characteristic of the disease (Sellon, 1993).

Infection with EIAV shortens the lifespan of circulating red blood cells to about 38 days (McGuire *et al.*, 1969). EIAV also have suppressive effect on erythroid series cell in bone marrow (Swardson *et al.*, 1992). The anemia caused by EIAV is not wholly due to the immune response of the host, but with severe

combined immunodeficiency of the infection (Perryman *et al.,* 1988). EIAV does not affect megakaryocytes and the suppressive effect of infection is due to at least in part to alpha and beta tumor necrosis factors (Crawford et *al.,* 1996).

2.8 Clinical Findings

An incubation period of two to four weeks is usual in natural outbreaks of equine infectious anemia. Outbreaks usually follow a pattern of slow spread to susceptible horses after the introduction of an infected animal. After infection, the equine experiences three distinct disease phases: acute, chronic and inapparent carrier (Roberts and Lucas, 1987).

Occasionally the initial attack is mild and may be followed by rapid clinical recovery. As a rule there is initial anorexia, depression, profound weakness, and loss of condition. Ataxia is a prominent sign in many cases and in some is recorded as the only clinical abnormality (McClure *et al.*, 1982). There is intermittent fever (up to 41°C), which may rise and fall rapidly, sometimes varying as much as 1°C within 1 hour. Jaundice, oedema of the ventral abdomen, the prepuce and legs, and petechial hemorrhages in the mucosa, especially under the tongue and in the conjunctiva, may be observed. Pallor of the mucosa does not occur in this early stage and they tend to be congested and oedematous (Clabough, 1990).

There is a characteristic increase in rate and intensity of the heart sounds, which are greatly exacerbated by moderate exercise. Myocarditis, manifested by tachycardia and arrhythmia, are described as being diagnostic. Respiratory signs are not marked, there is no dyspnea until the terminal stages, but there may be a thin